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The Inhibition of NF-κB Activation Pathways and the Induction of Apoptosis by Dithiocarbamates in T Cells Are Blocked by the Glutathione Precursor N-Acetyl-L-Cysteine

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Nuclear factor-κB regulates genes that control immune and inflammatory responses and are involved in the pathogenesis of several diseases, including AIDS and cancer. It has been proposed that reactive oxygen intermediates participate in NF-κB activation pathways, and compounds with putative antioxidant activity such as N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) have been used interchangeably to demonstrate this point. We examined their effects, separately and combined, on different stages of the NF-κB activation pathway, in primary and in transformed T cells. We show that NAC, contrary to its reported role as an NF-κB inhibitor, can actually enhance rather than inhibit IκB degradation and, most importantly, show that in all cases NAC exerts a dominant antagonistic effect on PDTC-mediated NF-κB inhibition. This was observed at the level of IκB degradation, NF-κB DNA binding, and HIV-LTR-driven reporter gene expression. NAC also counteracted growth arrest and apoptosis induced by dithiocarbamates. Antagonistic effects were further observed at the level of jun-NH_2-terminal kinase, p38 and ATF-2 activation. Our findings argue against the widely accepted assumption that NAC inhibits all NF-κB activation pathways and shows that two compounds, previously thought to function through a common inhibitory mechanism, can also have antagonistic effects.

Key words: Antioxidant / Disulfiram / Pyrrolidine dithiocarbamate / Reactive oxygen intermediates / T cell transformation / Theileria.

Introduction

NF-κB binds to decameric κB sites which are found in the promotors and enhancers of genes that are involved in inflammation, immune responses, disease, and apoptosis (Baeuerle and Henkel, 1994; Siebenlist *et al*., 1994; Baldwin, 1996). Consequently, extensive efforts are being invested in identifying steps in the NF-κB activation pathway that could be blocked in a targeted manner (Poli and Fauci, 1992; Blackwell *et al*., 1996; Sakurada *et al*., 1996). NF-κB is activated by a wide array of agents including cytokines, oxidants such as H_2O_2, phorbol esters, certain phosphatase inhibitors such as okadaic acid (OA), and UV or ionising radiation. Different receptors, among them members of the tumour necrosis factor receptor family that participate in apoptosis (Nagata, 1997), also activate NF-κB. In addition, NF-κB may participate in the pathogenesis of diseases caused by viruses and bacteria (reviewed in Baeuerle and Henkel, 1994; Baldwin, 1996). More recently, the list of NF-κB inducers has been extended to parasites such as *Toxoplasma* (Gazzinelli *et al*., 1996), *Plasmodium* (Schofield *et al*., 1996) and *Theileria* (Ivanov *et al*., 1989; Palmer *et al*., 1997) which interfere with host cell signalling pathways.

The NF-κB/Rel family of proteins consists of p50 (NFκB1), p52 (NFκB2), p65 (RelA), c-Rel and RelB (reviewed in Verma *et al*., 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996). These proteins share a conserved 'Rel homology domain' through which they can form homodimers or heterodimers which bind DNA. In most cell types, NF-κB is sequestered in the cytoplasm in a latent form in association with inhibitory IκB proteins, of which IκBα and IκBβ are the best characterised. Activation of NF-κB has been shown to involve signal-induced IκB phosphorylation by IκB kinases (reviewed in Stancovski and Baltimore, 1997). IκB phosphorylation and subsequent ubiquitination targets the protein for proteosomal degradation (Palombella *et al*., 1994; Traenckner *et al*., 1994; DiDonato *et al*., 1996). Ubiquitination-independent IκB degradation (Lee *et al*., 1997a) also occurs, however, and may be involved in regulating basal IκB turnover (Krappmann *et al*., 1996).

Numerous studies suggest that reactive oxygen species (ROS) serve as common intracellular mediators of IκB degradation and subsequent NF-κB activation (Schulze Osthoff *et al*., 1993; Baeuerle and Henkel, 1994; Baeuerle *et al*., 1996; Sen and Packer, 1996; Flohé *et al*., 1997). Consistent with this hypothesis, it has been shown that a variety of structurally diverse antioxidants are capable of inhibiting NF-κB activation. A number of studies, however, also suggest that not all NF-κB activation pathways involve ROS, as judged by their resistance to antioxidants (Suzuki *et al*., 1994; Brennan and O'Neill, 1995;

Flohé *et al*., 1997). N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) are two compounds that have been used extensively as NF-κB inhibitors (Schreck *et al*., 1992; Meyer *et al*., 1994). NAC, a glutathione (GSH) precursor and ROS scavenger, inhibits NF-κB activation induced by a range of stimuli in several, but not all, cell types (Schreck *et al*., 1991; Meyer *et al*., 1993). PDTC, a dithiol compound often used because of its reported antioxidant activity, is a more consistent inhibitor of NF-κB which appears to act more independently of the activating agent and cell line used (Schreck *et al*., 1991; Galter *et al*., 1994; Mihm *et al*., 1995). PDTC has recently also been shown to induce G1 cell-cycle arrest and apoptosis in malignant cell lines and to enhance the anti-tumour efficacy of chemotherapeutic agents, prompting the proposal that PDTC may find application in combination therapy (Chinery *et al*., 1997).

The fact that NAC and PDTC can independently inhibit NF-κB activation has been used in support of a general model in which NF-κB activation is governed by oxidative stress (Schreck *et al*., 1991). Evidence is emerging, however, that the major mode of inhibitory action of PDTC may not be restricted to its antioxidant activity, but could also include metal ion chelating (Bessho *et al*., 1994; Brennan and O'Neill, 1995) and even pro-oxidant properties (Nobel *et al*., 1995; Brennan and O'Neill, 1996b; Pinkus *et al*., 1996). In this context, PDTC-dependent activation of the transcription factor AP-1 was shown to depend on its pro-oxidant activity and could be blocked by anti-oxidants, including GSH and NAC, its synthetic precursor (Pinkus *et al*., 1996). In the present study, we investigated whether such antagonistic effects also existed at the level of the NF-κB activation in primary lymph node (LN) T cells and on induced and constitutive NF-κB activation in transformed T cells. We report that the GSH precursor, NAC, which is generally accepted as an antioxidant inhibitor of NF-κB, can actually enhance NF-κB activation rather than inhibit it, and, more importantly, we demonstrate that the inhibitory effects of dithiol compounds such as PDTC and disulfiram on NF-κB activation are neutralised completely by NAC. This effect can be observed at the level of IκBα and IκBβ degradation, NF-κB DNA binding activity, NF-κB transcriptional activity and dithiocarbamate-induced growth arrest and apoptosis. Our findings demonstrate that these classical NF-κB inhibitors, previously thought to function through a common antioxidant mechanism, can have completely diverging and antagonistic effects on NF-κB activation pathways.

Results

NAC Enhances Basal IκB Degradation

IκBa, in particular the uncomplexed form, is inherently unstable and undergoes a continuous turnover in the absence of NF-κB-inducing signals (Miyamoto *et al*., 1994; Krappmann *et al*., 1996). This is most apparent when *de novo* synthesis is blocked by the protein synthesis inhibitor cycloheximide (CHX). When resting LN T cells were cultured in the presence of CHX (Figure 1A), basal levels of IκBα turnover could be demonstrated by a modest reduction in the amounts of IκBα protein detected by Western blot analysis. Whereas PDTC treatment showed no effect, NAC actually enhanced IκBα turnover, indicating that the two compounds affect basal IκBα turnover differently. This finding was surprising, since NAC, as an antioxidant and ROS scavenger, was expected to inhibit rather than enhance IκBα degradation.

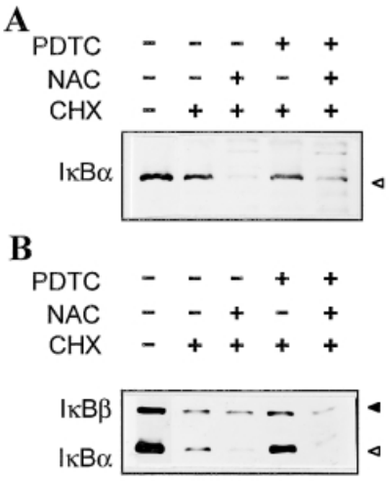

Fig. 1 Effect of NAC and PDTC on IκB Degradation in Resting LN T Cells and Transformed Tpi T Cells.
(A) Basal IκBα turnover in LN T cells is enhanced by NAC. LN T cells were cultured in the presence or absence of CHX to inhibit de novo IκBα synthesis and treated with NAC or PDTC, as indicated. Whole cell extracts (15 μg/lane) were examined by immunoblot analysis using anti-IκBα Ab. The position of IκBα is indicated by an open triangle.
(B) Effect of NAC and PDTC on constitutive IκB degradation in Tpi T cells. Tpi T cells were cultured in the presence of CHX, NAC or PDTC as indicated and whole cell extracts (20 μg/lane) examined by immunoblot analysis using anti-IκBα and anti-IκBβ Ab together. The positions of IκBα and IκBβ are indicated by open triangle and closed triangles, respectively. The upper open triangle marks phosphorylated IκBα.

NAC Blocks PDTC-Mediated Inhibition of IκB Degradation

In order to investigate whether this observation could be confirmed in cells in which the NF-κB activation pathway is constitutively activated, we next tested the effect of NAC and PDTC on IκB degradation in transformed T cells. As a source of transformed T cells, we used *Theileria parva*-infected T cells (Tpi T cells) which proliferate continuously and behave as persistently activated T cells (Dobbelaere *et al*., 1988; Eichhorn *et al*., 1993; Galley *et al*., 1997). In Tpi T cells, NF-κB is constitutively activated, involving the continuous degradation of both IκBα and IκBβ and nuclear translocation of several members of the NF-κB/Rel family. Steady-state levels of IκB were not sig-

nificantly affected by NAC or PDTC treatment except for a slight reduction in the amount of phosphorylated IκBα in PDTC-treated cells (data not shown). Blocking *de novo* protein synthesis in Tpi T cells by treatment with CHX for 4 h resulted in decreased levels of both IκBα and IκBβ compared to untreated cells (Figure 1B), reflecting the constitutive degradation of IκB (Palmer *et al*., 1997). As was observed for basal degradation in primary LN T cells, NAC enhanced constitutive IκBα degradation. Treatment with PDTC clearly prevented IκB degradation. Furthermore, in the presence of NAC, the inhibition of IκB degradation by PDTC was totally abrogated, demonstrating that the antioxidant NAC can block the effect of PDTC at the level of IκB degradation. OA is a strong activator of NF-κB and induces the rapid phosphorylation and degradation of IκB (Rieckmann *et al*., 1992; Menon *et al*., 1995), most likely by blocking protein phosphatase 2A which inactivates IκB kinase (DiDonato *et al*., 1997). In earlier work, it has been proposed that OA induces IκB degradation downstream of the anti-oxidant-sensitive step in the NF-κB activation cascade (Suzuki *et al*., 1994; Sen and Packer, 1996). When resting LN T cells were stimulated with OA, IκBα was phosphorylated and rapidly degraded (Figure 2A) and in agreement with earlier reports (Suzuki *et al*., 1994; Sen and Packer, 1996), IκBα degradation was not inhibited by NAC, PDTC or a combination of both. OA-induced IκB degradation was also restricted to IκBα and no IκBβ degradation could be detected. In the transformed Tpi T cells, OA also induced the complete degradation of IκBα (Figure 2B). In contrast to resting LN T cells in which IκBβ levels were not affected by OA, significant IκBβ degradation could be detected in transformed Tpi T cells. Only a faint IκBβ band (small closed triangle) could be detected upon OA treatment, and degradation was again enhanced by NAC. PDTC largely prevented the OA-induced degradation of IκBβ, but not that of IκBα. The stabilisation of IκBβ conferred by PDTC was completely prevented by NAC, demonstrating that the antagonistic effect of NAC on PDTC can occur both at the level of constitutive IκB turnover and OA-induced IκB degradation. Triggering of T cell surface receptors for TNF, IFNγ, IL1β or stimulation of CD95/Fas or CD28 generates ceramide via hydrolysis of sphingomyelin by sphingomyelinases (reviewed in Ballou *et al*., 1996). Ceramide is an important signal transducer of NF-κB activation. N-acetylsphingosine, a membrane-permeable synthetic form of ceramide, was used to test the effect of PDTC and NAC on ceramide-induced IκB degradation in Tpi T cells. Within 1 h of ceramide treatment, IκBα was strongly reduced and IκBβ had all but disappeared. PDTC inhibited the ceramide-induced degradation of both IκBα and IκBβ. NAC further enhanced IκB degradation and the PDTC-mediated inhibition of IκB degradation was again prevented by NAC (not shown).

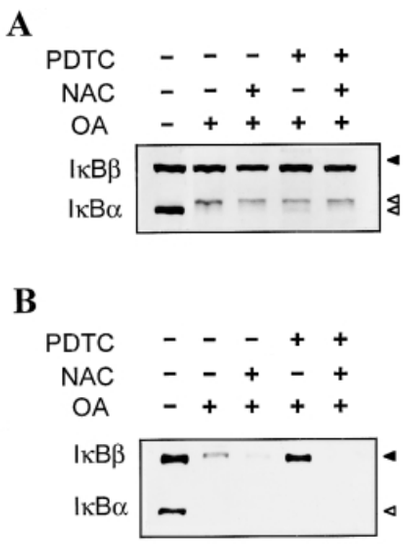

Fig. 2 Effect of NAC and PDTC on OA-Induced IκB Degradation in Resting LN T Cells and Transformed Tpi T Cells.
(A) NAC and PDTC have no effect on OA-induced IκBα degradation in resting LN T cells. LN T cells were stimulated with OA in the presence or absence NAC or PDTC as indicated. The position of IκBα (lower open triangle) and its phosphorylated form (upper open triangle) and the position of IκBβ (closed triangle) are indicated.
(B) Tpi T cells were stimulated with OA in the presence or absence of NAC and PDTC and protein extracts were analysed for IκB levels as above.

The Antagonistic Effect of NAC on PDTC Is Also Evident at the Level of NF-κB DNA Binding and Transcriptional Activity

In previous work we have shown by electrophoretic mobility shift assay (EMSA) that *T. parva* induces NF-κB activation resulting in the appearance of different κB binding complexes in the nucleus (Ivanov *et al*., 1989). NF-κB transcriptional activity in Tpi T cells was also shown to be resistant to NAC and only partly reduced by treatment with PDTC (Palmer *et al*., 1997). Consistent with these observations, NAC had no inhibitory effect on NF-κB DNA binding activity (Figure 3); on the contrary, in agreement with the enhanced IκB degradation induced by NAC, DNA binding was often moderately increased in the presence of

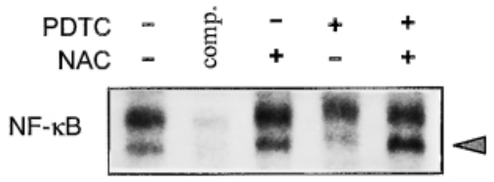

Fig. 3 Influence of NAC and PDTC on NF-κB DNA Binding Activity.
NF-κB translocation and DNA binding in Tpi T cells is partially inhibited by PDTC, but not by NAC, and NAC counteracts PDTC-mediated inhibition. Tpi T cells were left untreated or treated for 5 h with PDTC and NAC as indicated. Nuclear lysates were prepared from 10^7 cells and NF-κB binding to [^{32}P]-labelled oligonucleotides representing consensus κB recognition sites determined by EMSA. The arrowhead indicates the NF-κB complex which is most affected by PDTC. The lane labelled 'comp', shows an assay in which an excess of unlabelled oligonucleotide included in the EMSA as a control for binding specificity.

NAC. PDTC treatment resulted in a partial inhibition of DNA binding, affecting predominantly the faster migrating NF-κB complex (marked by an arrowhead). NAC completely restored NF-κB DNA binding in PDTC-treated cells as shown by the reappearance of the lower complex. Treatment of Tpi cells with OA resulted in increased NF-κB binding activity (not shown) and consistent with the observations made for IκB, NAC did not inhibit either quantitative or qualitative changes. Treatment with PDTC, on the other hand, resulted in reduced NF-κB DNA binding activity, but this effect was at least partly blocked when cells were simultaneously treated with NAC (data not shown).

To test whether the antagonistic effects of NAC on the activity of PDTC could also be demonstrated at the level of NF-κB transcriptional activity, transient transfection experiments were performed using the reporter plasmid HIV-CAT. In this plasmid, expression of the chloramphenicol acetyl transferase (CAT) gene is placed under the control of the two κB binding sites within the HIV-LTR. As shown before for Tpi T cells (Palmer *et al*., 1997), the constitutive NF-κB transcriptional activity, monitored by CAT activity, was not inhibited by NAC (Figure 4A), but was reduced by 62% when cells were treated with PDTC. No reduction in CAT activity could be observed, however, when cells were treated simultaneously with both compounds. The effects of both compounds on basal NF-κB transcriptional activity in Jurkat T cells was identical to that observed in Tpi T cells, with CAT activity reduced by 57% in the presence of PDTC (data not shown). In Tpi T cells, NF-κB is maximally induced and can not be activated further. Stimulation of Jurkat T cells with TPA and ionomycin, however, resulted in a 4.8-fold induction above basal activity (Figure 4B), which was completely inhibited by PDTC. NAC did not interfere with the induction of

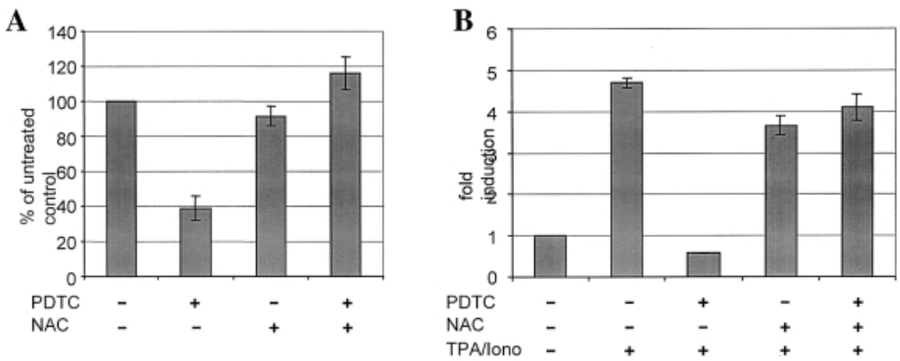

Fig. 4 PDTC-Mediated Inhibition of NF-κB Dependent Reporter Gene Expression Is Abolished by NAC.
(A) Tpi T cells, in which NF-κB is constitutively activated, were transfected with the plasmid HIV-CAT, in which CAT expression is stimulated through two κB binding sites. Transfected cells were either untreated or incubated with PDTC and/or NAC for 24 h prior to determination of CAT activity. Results represent one of at least three separate experiments and are expressed as % of CAT activity in untreated HIV-CAT-transfected cells. Bars represent the mean value (± S.D., *n* =3).
(B) Effect of NAC and PDTC on NF-κB-dependent reporter gene expression in HIV-CAT-transfected Jurkat T cells, induced by treatment with TPA and ionophore. Results from one of at least three experiments are presented as fold induction compared to unstimulated Jurkat T cells. Bars represent the mean value (± S.D., *n* = 3).

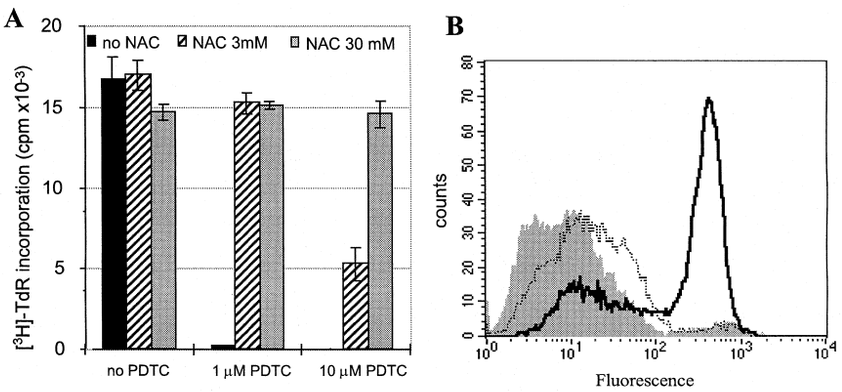

Fig. 5 NAC Prevents PDTC-Induced Growth Arrest and Apoptosis.
(A) Jurkat T cells were cultured overnight in the presence or absence of PDTC and NAC at the indicated doses. Cells were labelled with [methyl-^{3}H]thymidine ([^{3}H]TdR) the last 5 h of culture and [^{3}H]TdR incorporation measured. Data are presented as cpm $\times 10^{-3}$ per 4×10^4 cells seeded. Error bars represent 1 SD of the mean of values from triplicate cultures. (B) Jurkat T cells were cultured for 16 h in the presence of PDTC (10 μg/ml) alone (solid line) or PDTC and NAC (30 mM) together (dotted line), and analysed by flow cytometry for the binding of fluoresceine-labelled annexin-V. The grey profile shows annexin-V binding to control cells treated with NAC alone. Propidium iodide DNA staining was used to exclude necrotic cells from the analysis.

NF-κB and again prevented PDTC-mediated inhibition of NF-κB activation. These data confirm that the antagonistic effect of NAC on PDTC-mediated inhibition of NF-κB activation can be observed at several levels of the activation cascade.

NAC Counters PDTC-Induced Growth Arrest and Apoptosis in Transformed T Cells

There are conflicting reports on the effect of PDTC on proliferation and apoptosis. PDTC has been reported to induce apoptosis in thymocytes (Nobel *et al*., 1995) and tumour cells (Chinery *et al*., 1997; Giri and Aggarwal, 1998). On the other hand, PDTC has also been shown to protect cells from apoptosis (Albrecht *et al*., 1994; Bessho *et al*., 1994; Mihm *et al*., 1995). The proliferation of both Tpi and Jurkat T cells was monitored by [methyl-^{3}H]thymidine incorporation and found to be potently inhibited by micromolar amounts of PDTC. In both cases, however, the PDTC-induced proliferation arrest was reversed by cotreatment with NAC (Figure 5A). In order to determine whether growth arrest was followed by apoptosis, the expression of phosphatidylserine was also monitored. During the induction of apoptosis, changes occur at the cell surface, including the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer. Annexin-V is a PS-binding protein which binds with high affinity to PS and can be detected by flow cytometry (Verhoven *et al*., 1995). When Jurkat T cells were treated for 16 h with PDTC, the majority of cells exposed PS on their surface (Figure 5B). A clear reduction in annexin-V binding could be observed, however, when cells were cultured in the presence of both PDTC and NAC, demonstrating that NAC is capable of preventing PDTC-induced apoptosis in Jurkat T cells. Identical results were obtained for Tpi T cells (not shown).

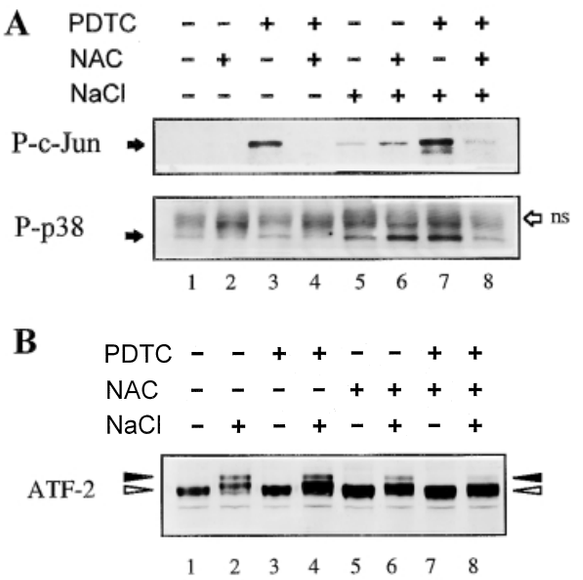

Fig. 6 Effects of NAC and PDTC on JNK, p38 and ATF-2 Activation.
(A) Effect in resting LN T cells and LN T cells subjected to osmotic shock. Resting LN T cells (lanes 1–4) or LN T cells subjected to osmotic shock (0.2 M NaCl for 20 min; lanes 5–8) were cultured in the presence or absence of PDTC and NAC. Nuclear extracts were analysed by immunoblotting, using Ab specific for phospho-c-Jun (top panel) or phospho-p38 (lower panel). Closed arrows point at the specific phospho-c-Jun and phospho-p38 bands. In the lower panel, the position of a non-specific band (ns) is indicated by an open arrow.
(B) Effect of NAC and PDTC on ATF-2 activation in Tpi T cells subjected to osmotic shock. Immunoblot analysis of extracts prepared from Tpi T cells that were either untreated or submitted to osmotic shock (even numbers) in the presence or absence of NAC and PDTC. The filter was probed with an anti-ATF-2 Ab; unphosphorylated and phosphorylated ATF-2 are indicated by open and closed arrowheads, respectively.

Effects of NAC and PDTC on jun-NH_2-Terminal Kinase (JNK), p38 and ATF-2

Many stimuli that induce NF-κB also activate the MAP kinase family members JNK and p38. Previous work has shown that NAC and PDTC can both induce JNK in Jurkat T cells (Gomez del Arco *et al*., 1996). We tested whether this also applied to resting LN T cells and whether NAC could block PDTC-induced effects. Basal JNK and p38 activity and their induction by osmotic shock were examined. Nuclear extracts from LN T cells were subjected to Western blot analysis, using antibodies (Ab) directed against phosphorylated forms of c-Jun and p38. Phosphorylation of c-Jun, detectable by anti-phospho-c-Jun Ab, reflects JNK activity; likewise, p38 activation is accompanied by specific p38 autophosphorylation. PDTC treatment resulted in increased c-Jun phosphorylation in resting LN T cells and also in cells that were subjected to mild osmotic shock (Figure 6A, lanes 3 and 7). NAC weakly super-induced JNK activation only in NaCl-treated cells. PDTC-mediated JNK activation, however, was potently inhibited when NAC was present (compare lanes 3 and 4, lanes 7 and 8). In Tpi T cells, in which JNK is constitutively activated (Galley *et al*., 1997), superinduction of JNK by PDTC was also blocked by NAC (data not shown). A basal level of p38 activity could be detected in resting LN T cells which was not observed in the presence of NAC. PDTC treatment did not result in additional p38 activity. In LN T cells subjected to osmotic shock, p38 was clearly upregulated and this was enhanced by NAC or PDTC treatment. In the presence of both compounds, however, the levels of phospho-p38 were markedly reduced.

The JNK and p38 pathways have been reported to regulate the phosphorylation and activation of transcription factors such as AP-1 and ATF-2 (Gupta *et al*., 1995) (reviewed in Whitmarsh and Davis, 1996). It has previously been described that PDTC-induced AP-1 activation can be inhibited by glutathione and NAC (Pinkus *et al*., 1996). We investigated whether this also applied to ATF-2. Activation of ATF-2 is accompanied by changes in its phosphorylation pattern, resulting in the appearance of additional bands with reduced electrophoretic mobility. Total cellular extracts from Tpi and resting LN T cells were

subjected to immunoblot analysis, using Ab specific for ATF-2. Marked changes in ATF-2 phosphorylation patterns could be observed in Tpi or LN T cells that were subjected to osmotic shock (shown for Tpi T cells in Figure 6B, even numbers). NAC treatment did not induce any changes, but ATF-2 phosphorylation was more pronounced in the presence of PDTC (Figure 6B, compare lanes 2 and 4) and the pattern of intensity of the different bands was also altered. In the presence of both NAC and PDTC, however, ATF-2 phosphorylation induced by osmotic shock was significantly reduced (Figure 6B, compare lanes 4 and 8). Thus, PDTC can enhance both AP-1 (Pinkus *et al*., 1996) and ATF-2 activation and, in both cases, these effects are reversed by NAC.

NAC Also Counteracts the Inhibitory Effects Induced by Another Thiol, Disulfiram

Finally, we determined whether NAC could counteract the action of dithiol compounds other than PDTC. Disulfiram, a drug used for alcohol aversion therapy, is also a potent inhibitor of NF-κB (Schreck *et al*., 1992). Disulfiram treatment of Tpi T cells resulted in a strong inhibition of NF-κB DNA binding (Figure 7A). Although the pattern of inhibition was different from that caused by PDTC, disulfiram-mediated NF-κB inhibition was completely prevented by NAC. Inhibition of NF-κB activation by non-thiol compounds such as *o*-phenanthroline and desferrioxamine, on the other hand, was not blocked by NAC (data not shown). Transfection studies with HIV-CAT plasmid showed that disulfiram treatment resulted in a significant reduction in CAT activity (Figure 7B) which was reversed by NAC. As was the case for PDTC, disulfiram caused rapid growth arrest and apoptosis of Jurkat and Tpi T cells, which was counteracted when NAC was added to the culture medium. Figure 7C shows a typical experiment using Tpi T cells in which proliferation was monitored by measuring [methyl-^{3}H]thymidine incorporation. Disulfiram potently blocked proliferation, but this did not occur in the presence of NAC. An identical pattern was again observed for Jurkat T cells (not shown).

Discussion

Lack of Susceptibility to Inhibition by NAC

Many inducers of NF-κB activation are known to result in the generation of intracellular ROS (Schreck *et al*., 1992; Meyer *et al*., 1994) (reviewed in Schreck and Baeuerle,

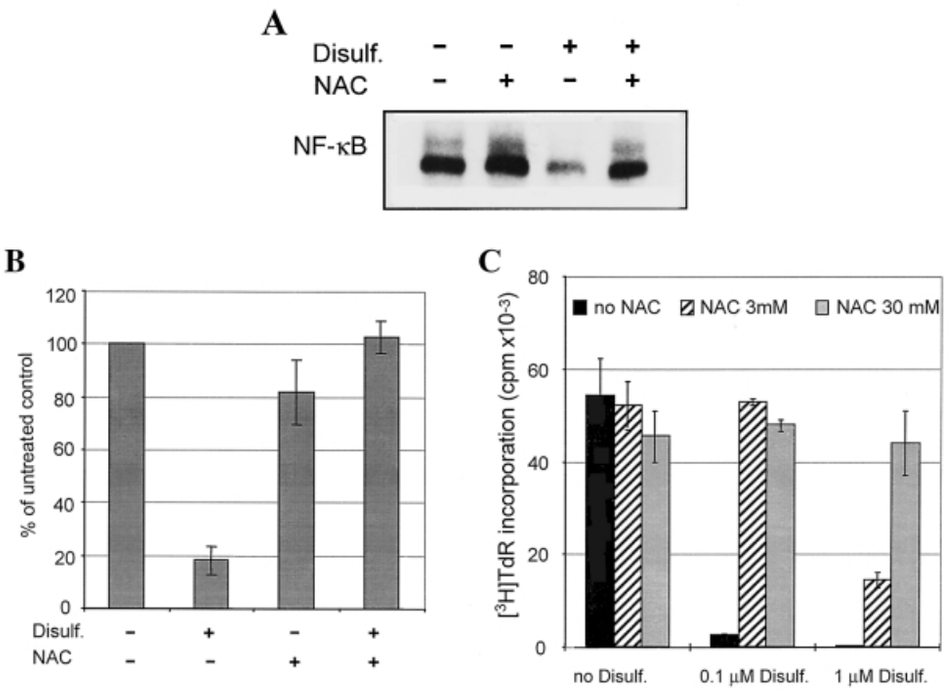

Fig. 7 NAC Blocks Disulfiram-Mediated Inhibition of NF-κB and Disulfiram-Induced Growth Arrest.
(A) NAC prevents disulfiram-mediated inhibition of NF-κB activation. Tpi T cells were left untreated or treated with disulfiram (1 μM) and/or NAC (30 mM) for five hours. Nuclear extracts were examined by EMSA as described for Figure 3.
(B) The inhibition of NF-κB transcriptional activity by disulfiram is prevented by NAC. Tpi T cells were transfected with the plasmid HIV-CAT. Constitutive NF-κB transcriptional activity was monitored in transfected cells that were either left untreated or incubated with disulfiram (1 μM) and/or NAC (30 mM) for 24 hours prior to determining of CAT activity. One of two separate experiments is shown and results are expressed as % of CAT activity in untreated control cells. Bars represent the mean value (± S.D., $n = 3$).
(C) Disulfiram-induced arrest of proliferation is prevented by NAC. Tpi T cells were cultured for 24 h in the presence or absence of disulfiram and NAC at the indicated doses. Cells were labelled with [methyl-^{3}H]thymidine ([^{3}H]TdR) for the last 5 h of culture and incorporation measured by liquid scintillation counting. Data are presented as cpm $\times 10^{-3}$ per 4×10^4 cells seeded. Error bars represent 1 SD of the mean of values from triplicate cultures.

1994). This, together with the fact that NF-κB activation can be inhibited by antioxidants, has lead to the proposal that ROS serve as second messengers in most, if not all, NF-κB activation pathways (Schreck *et al*., 1991; Schreck and Baeuerle, 1994). In these studies, NAC and dithiols have been used interchangeably to underpin this theory. Thus, both PDTC and NAC have been shown to inhibit the synthesis of IL-6, IL-8, and GM-CSF induced by TNF or bacterial lipopolysaccharides (LPS) in human umbilical vein endothelial cells (Munoz *et al*., 1996) and similar observations were made in other systems (Lee *et al*., 1997b; Kikumori *et al*., 1998). Contrary to our findings, NAC has also been shown to inhibit NF-κB activation in a number of T cell lines (Dröge *et al*., 1991; Mihm *et al*., 1995). The concept that NF-κB is a sensor for oxidative stress has recently been challenged more directly (Li and Karin, 1999) and arguments against a general involvement of ROS in NF-κB activation are accumulating (Suzuki *et al*., 1994, 1995) (reviewed in Flohé *et al*., 1997; Li and Karin, 1999). NAC failed to inhibit the activation of NF-κB by classical NF-κB-inducing agents such as IL-1, TNF and TPA in some cell lines, but not in others (Brennan and O'Neill, 1995; Bowie *et al*., 1996). Likewise, in the T lymphocytic cell line, CEM, NF-κB activation by PMA was resistant to NAC (Legrand Poels *et al*., 1997). These findings indicate that the ROS model of NF-κB activation and sensitivity to NAC is restricted to certain cell types. Furthermore, H_2O_2 does not invariably activate NF-κB (Israel *et al*., 1992; Anderson *et al*., 1994; Brennan and O'Neill, 1995) and studies involving the transient overexpression of catalase suggest that H_2O_2 may not always serve as a second messenger in NF-κB activation (Suzuki *et al*., 1995). Defining the exact role of H_2O_2 (or other ROS) as an integratory secondary messenger for different T cell signals has indeed been complicated by the fact that various T cell lines and peripheral blood T cells can differ markedly – also within one cell line – in the levels of NF-κB activation induced by oxidant stress (Ginn Pease and Whisler, 1998). Various factors appear to regulate the responsiveness, since it has been shown that optimal NF κB-mediated transcriptional responses in Jurkat T cells exposed to oxidative stress are dependent on intracellular glutathione and costimulatory signals (Ginn Pease and Whisler, 1996). The nature of the stimulus may also play a role. For instance, it has been shown that NAC and PDTC were also not able to block daunomycin-induced NF-κB activation or p53 induction, indicating that ROI are not involved in the cellular response to daunomycin stimulation (Hellin *et al*., 1998).

A consistent observation in our work was the general lack of inhibitory activity observed for NAC in the systems that were tested. Assuming that NAC is an antioxidant which indeed blocks the effects of ROS, our observations strengthen the concept that the ROS-dependence of NF-κB activation may not be as strict or widespread as previously proposed (Schulze Osthoff *et al*., 1997) and also argue in favour of cell-type restriction. Moreover, and contrary to expectations, NAC enhanced rather than inhibited IκB degradation. Indeed, we found the basal IκBα turnover in resting LN T cells, the constitutive degradation in transformed Tpi T cells, and OA- or ceramide-induced IκB degradation to be enhanced in the presence of NAC. While this observation may be unexpected, NAC-mediated NF-κB activation is not without precedent. It has recently been reported that treatment of macrophages with NAC resulted in increased NF-κB activity which correlated with an up-regulation of HIV production (Nottet *et al*., 1997).

NAC Blocks PDTC-Mediated Inhibition of NF-κB

One of the salient observations of our work was that NAC consistently blocked the PDTC-mediated inhibition of the NF-κB activation pathway, irrespective of the way in which NF-κB activation was induced. This could be observed at the level of IκB degradation, NF-κB DNA binding and transcriptional activity. These findings are not compatible with the proposed common mode of action for PDTC and NAC, in which both compounds are thought to inhibit NF-κB activation through their antioxidant activity (Meyer *et al*., 1993, 1994). Does PDTC really function as an antioxidant? Dithiocarbamates can induce a variety of other biological effects in mammalian cells (Sen and Packer, 1996; Flohé *et al*., 1997). A number of studies suggest that the inhibition of NF-κB by PDTC and other dithiocarbamates occurs through their metal ion chelating properties (Bessho *et al*., 1994; Brennan and O'Neill, 1995; Nobel *et al*., 1995) and several other, non-thiol metal chelators such as *o*-phenanthroline and desferrioxamine also inhibit NF-κB (Schreck *et al*., 1992). Nevertheless, we found that the inhibitory effect of the latter two compounds was not blocked by NAC, suggesting that NAC probably does not act by blocking the potential metal chelating properties of PDTC. In addition, it has been proposed that PDTC may cause GSH oxidation, resulting in GSSG accumulation, by a non-radical mechanism (Ziegler, 1991). Brennan and O'Neill (1996a) also reported PDTC pro-oxidant activity, resulting in increased levels of GSSG which can affect the binding of NF-κB to DNA. In addition, PDTC pro-oxidant, rather than antioxidant, activity has been shown to be responsible for inducing AP-1 activation (Pinkus *et al*., 1996). NF-κB activation apparently requires optimal levels of GSSG (Dröge *et al*., 1994) which is regulated in a complex manner. At low GSSG levels, T cells cannot optimally activate NF-κB, whereas high GSSG levels inhibit the DNA binding activity of NF-κB. The effects of GSSG, in turn, are antagonized by reduced thioredoxin (Dröge *et al*., 1994) which has distinct roles in the cytoplasm and in the nucleus (Flohé *et al*., 1997). Considered together, it is conceivable that NAC, as a precursor of GSH, counteracts the effects of increased GSSG levels induced by PDTC pro-oxidant activity, by restoring the intracellular redox equilibrium. Recent work, however, has shown that the effects of NAC on T cell apoptosis are not mediated by increased cellular glutathione (Jones *et al*., 1995). NAC, in its capacity of a thiol, may also counter the pro-oxidant activity of PDTC more directly. That this may indeed be the case is strongly supported by recent observations made in our

laboratory showing that both L-cysteine and D-cysteine (which can not be incorporated into GSH) on their own are capable of blocking growth inhibition and apoptosis induced by PDTC in Tpi and Jurkat T cells (VH unpublished observation). Our observations also point towards a number of differences in the regulation of the IκBα and IκBβ degradation pathways. As has also previously been observed for Jurkat T cells and U937 monocytic cells (Suzuki *et al*., 1994; Sen and Packer, 1996), OA-induced degradation of IκBα could not be inhibited by PDTC in Tpi T cells. OA-induced degradation of IκBβ, on the other hand, was blocked (Figure 2B), demonstrating a differential sensitivity of the IκBα and IκBβ degradation pathways to PDTC-mediated inhibition. We also observed that OA was capable of inducing IκBβ degradation in the transformed Tpi T cells, but not in resting LN T cells. This is reminiscent of the observations by Menon *et al*. (1993), who showed that OA treatment failed to induce nuclear translocation of NF-κB in several primary cell lines, whereas rapid induction by OA could readily be detected in their SV40-transformed counterparts. Both sets of observations support the concept that the regulation of IκB degradation pathways can differ depending on the state of activation of the cell.

NAC Blocks Apoptosis Induced by PDTC

Tpi and Jurkat T cells proved highly sensitive to PDTC and disulfiram, undergoing rapid cell cycle arrest and apoptosis. The fact that these processs are averted by NAC points towards different modes of action.

In other systems, PDTC and NAC either induce or inhibit apoptosis, depending on the cell line and the stimulus (Bessho *et al*., 1994; Talley *et al*., 1995; Kaneto *et al*., 1996; Ivanov *et al*.,1997). In cancer cells, PDTC can induce apoptosis and it also potentiates the activity of chemotherapeutic agents at doses that are not toxic to a range of untransformed epithelial cells (Chinery *et al*., 1997). For this reason, clinical trials for the treatment of colorectal cancer and other solid tumours have been proposed, in which PDTC could be used in combination with chemotherapeutic agents (Chinery *et al*., 1997). Our observations suggest that the potentiating, beneficial effects of PDTC during such treatment may not be possible in the presence of a drug such as NAC.

Interestingly, the anti-apoptotic effect of NAC is not specific for apoptosis induced by PDTC. In recent work, we demonstrated that NAC also blocκs apoptosis induced by the alkylating agent N-α-tosyl-L-phenylalanine chloromethyl ketone (Heussler *et al*., 1999a), which inhibits the NF-κB activation pathway in Tpi T cells by blocking IκB phosphorylation (Heussler *et al*., 1999b).

Effects of NAC and PDTC on Other Signalling Pathways

AP-1, another key transcription factor, is also subject to redox regulation and its activation is also affected by PDTC and NAC (Meyer *et al*., 1993; Munoz *et al*., 1996; Pinkus *et al*., 1996; Yokoo and Kitamura, 1996). AP-1 transactivation is influenced by the intracellular thioredoxin and GSH status. PDTC, rather than inhibiting, induces AP-1-regulated gene expression. Such opposite effects on NF-κB and AP-1 activation have also been documented for H_2O_2, thioredoxin and a number of other compounds (Meyer *et al*., 1993; Galter *et al*., 1994; Schenk *et al*., 1994). Interestingly, Pinkus *et al*. (1996) demonstrated that PDTC-induced AP-1 activation is inhibited by pre-exposure of cells to GSH or antioxidants, including NAC. This is in line with our observations for the MAP kinase family members JNK and p38, and for the transcription factor ATF-2 which can be regulated by JNK and p38 (Gupta *et al*., 1995; Raingeaud *et al*., 1995; van Dam *et al*., 1995; Wilhelm *et al*., 1995). Treatment with PDTC enhanced JNK activation and this was blocked by NAC. In cells subjected to osmotic shock, JNK and p38 activity were enhanced by both NAC and PDTC, but strongly reduced in the presence of both components. NAC and PDTC, applied separately, did not interfere with ATF-2 activation induced by osmotic shock. On the contrary, PDTC tended to enhance ATF-2 phosphorylation. When osmotic shock was applied in the presence of both compounds, however, a clear reduction in the levels of phosphorylated ATF-2 could be observed. Our findings thus show that NAC blocks the effects exerted by PDTC on different signalling pathways, be they inhibitory, as is the case for NF-κB, or stimulatory, as was found for AP-1 and ATF-2. It has been reported that NF-κB activation can be regulated by MEKK-1 (Hirano *et al*., 1996; Meyer *et al*., 1996; Lee *et al*., 1997a), a kinase which also controls the activation of JNK and p38. The physiological significance of MEKK1 vs. NIK or other kinases activators of IKK, however, has not yet been fully elucidated (Karin and Delhase, 1998).

Nevertheless, despite the fact that the NF-κB and JNK/p38 pathways may at one stage be regulated by a common upstream element, pathways further downstream clearly appear to differ in their sensitivity to NAC and PDTC (Pinkus *et al*., 1996).

NF-κB represents a prime target for therapeutic intervention since blocking NF-κB activation inhibits both the production of inflammatory cytokines (such as TNF-α, IL1-β and IL6) by immune and endothelial cells (Munoz *et al*., 1996) and the deleterious cellular changes induced by cytokines (Barnes and Adcock, 1997). NF-κB and AP-1 binding sites are also present in the promoter region of a number of genes that are directly involved in the pathogenesis of AIDS and cancer. The LTR of HIV-1 contains κB binding sites which may play a crucial role in regulating the latency of AIDS (Griffin *et al*., 1989) and one of the major consequences of NF-κB activation is HIV gene transcription. Antioxidants such as NAC, dithiocarbamates and a wide variety of chemically unrelated inhibitors have been shown to block NF-κB activation or transcription directed by the HIV-LTR (Roederer *et al*., 1990; Staal *et al*., 1993) suggesting a possible therapeutic value in the clinical treatment of HIV-infected patients (Lemarie *et al*., 1986; Reisinger *et al*., 1990; Sunderman, 1991; Dröge *et al*.,

1992; Roederer *et al*., 1992). As mentioned before, however, NAC can also enhance acute HIV-1 replication in monocyte-derived macrophages (Nottet *et al*., 1997) and we now show that NAC can also counteract the inhibitory effects of dithiol compounds on HIV-LTR-driven reporter gene expression in T cells. In conclusion, it is becoming more and more obvious that NF-κB responses, and the extent to which they can be inhibited, differ significantly according to cell type, stimulus or state of activation of the cell. At this stage, our observations are limited to NF-κB activation in primary LN T cells and transformed T cells involving only a limited number of stimuli. The consistency with which NAC inhibited the effects of dithiocarbamates, however, suggests that it may be worthwhile testing whether these finding also apply to other systems. From a clinical point of view, our findings also provide a compelling argument for the judicious choice of drugs in the design of NF-κB-based therapies.

Materials and Methods

Cell Lines and T Cell Cultures

The *T. parva*-infected (Tpi) T cell line TpM(803) is $CD4^+$, $CD8^-$, α/β TCR^+ and its characteristics and maintenance have been described previously (Dobbelaere *et al*., 1988). LN T cells were prepared as described previously (Mastro and Pepin, 1980).

Drug Treatment, Processing and Analysis

Chemical inhibitors or activators were added to the final concentrations as follows: cycloheximide (CHX; 177 nM; Sigma), pyrrolidine dithiocarbamate (PDTC; 100 μM for short-term treatment in EMSA and 10 μM for long-term treatment in transfection assays; Sigma), *N*-acetylcysteine (NAC; 30 mM or as indicated; Sigma), tetradecanoyl phorbol acetate (TPA; 3.3 nM; Sigma); ionomycin (2 μM; Sigma); okadaic acid (OA; 2 or 4 μM as indicated; LC laboratories); C2-ceramide (100 μM or as indicated; Calbiochem, Lucerne, Switzerland); disulfiram (100 μM for EMSA, 1 μM for transfection assays or as indicated for proliferation assays; Sigma). Treatment of Tpi or LN T cells with NAC and/or PDTC was for 5 h; CHX treatment of LN T cells was for 5 h; for treatment of Tpi T cells, CHX was added for the last 4 h of NAC/PDTC treatment. Tpi T cells were induced with OA (2 μM) for the last 90 min of NAC/PDTC treatment; LN T cells were induced with OA (4 μM) for the last 40 min; disulfiram was added for the last 90 min. Ceramide stimulation was carried out for the last hour of NAC/PDTC treatment.

Proliferation assays were carried out as follows: 1×10^5 cells/ml were seeded in 96 flat-bottom well plates and cultured for 24 h in the presence or absence of NAC, PDTC or disulfiram, as indicated. Cells were pulsed with [methyl-^{3}H]thymidine (0.2 μCi/well; 1 mCi = 37 MBq) for the last five hours and incorporation measured by liquid scintillation counting. Apoptosis was monitored using the Annexin-V-Fluos kit from Boehringer.

Cell Lysates and Immunoblot Analysis

Cells were collected and washed twice in ice-cold PBS, pH 7.4, and then processed for immunoblot analysis as described before (Palmer *et al*., 1997) . Rabbit anti-peptide polyclonal Ab with the following specificities were used: IκBα (C-terminal 21 amino acids; sc-371, Santa Cruz, Basel, Switzerland); IκBα (C-terminal 20 amino acids; sc-945); p105/p50 (C-terminal 20 amino acids; sc-114); RelA/p65 (C-terminal 20 amino acids; sc-372); ATF-2 (sc-187), phospho-c-Jun (SER63) II antibody (9261s, New England Biolabs, Allschwill, Switzerland) and phospho-specific p38 MAPK antibody (9211s, New England Biolabs). Bound primary Ab detected using horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (ECL, Amersham Corp).

Electrophoretic Mobility Shift Assay (EMSA), Cell Transfection and CAT Assays

EMSA was carried out using oligonucleotides (5′-AGTTGA-GGGGACTTTCCCAGGC-3′ and its complement) in which the consensus NF-κB binding site (underlined) is incorporated (Ivanov *et al*., 1989). Cells were treated with NAC or PDTC for 5 h. OA (4 μM) stimulation was carried out for 40 min, in the case of PDTC- or NAC-treated cells, during the last 40 min of treatment. Plasmids used for transfection have been described before (Ivanov *et al*., 1989). Plasmid –121/+232 HIV-CAT and control –76/+232 HIV-CAT were derived by modification of pBLCAT5 containing the bacterial chloramphenicol acetyltransferase (CAT) gene. In plasmid –121/+232 HIV-CAT (abbreviated HIV-CAT), the CAT gene is under control of an HIV-1 enhancer with two κB elements and the promoter region of the enhancer. Plasmid –76/+232 HIV-CAT (ΔHIV-CAT) is identical except for deletion of the region containing the κB elements. Cells were transfected by electroporation using standard protocols (2×10^7 cells/ml, 600 μl aliquots, 20 μg of plasmid DNA, 430 V, 250 Ω). After electroporation, cells were placed on ice for 5 min and then diluted in Leibovitz L15 medium and incubated at 37 °C for 24 h. Treated or untreated cells were incubated for an additional 24 h, collected and washed with PBS, and then lysed by freeze-thawing in a CAT assay buffer (100 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT). After centrifugation, the supernatant was collected, incubated for 6 min at 65 °C, then re-centrifuged. A 15 μl aliquot was assayed for CAT activity using 9.25×10^3 Bq (250 nCi) of [^{14}C]-chloramphenicol in CAT assay buffer supplemented with 100 μg bovine serum albumin and 2 mg/ml *n*-butyryl coenzyme A. Butyrylated chloramphenicol was extracted using xylene and radioactivity determined by liquid scintillation counting.

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